Bisphenol A stimulates human lung cancer cell migration via upregulation of matrix metalloproteinases by GPER/EGFR/ERK1/2 signal pathway

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Lung cancer is one of the leading causes of cancer deaths worldwide. Recent evidences indicated that bisphenol A (BPA), a wide contaminant with endocrine disrupting activity, could enhance the susceptibility of carcinogenesis. Although there are increasing opportunities for lung cells exposure to BPA via inhalation, there is no study concerning the effects of BPA on the development of lung cancer. The present study revealed that BPA less than 10^-4 M had limited effects on the proliferation of lung cancer A549 cells, however, BPA treatment significantly stimulated the in vitro migration and invasion of cells combing with the morphological changes and up regulation of matrix metalloproteinase-2 (MMP-2) and MMP-9. G-protein-coupled estrogen receptor (GPER), while not estrogen receptor α/β (ERα/β), mediated the BPA induced up regulation of MMPs. Further, BPA treatment induced rapid activation of ERK1/2 via GPER/EGFR. GPER/ERFR/ERK1/2 mediated the BPA induced up regulation of MMPs and in vitro migration of lung cancer A549 cells. In summary, our data presented here revealed for the first time that BPA can promote the in vitro migration and invasion of lung cancer cells via upregulation of MMPs and GPER/ERFR/ERK1/2 signals, which mediated these effects. This study suggested that more attention should be paid on the BPA and other possible environmental estrogens induced development of lung cancer.

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1. Introduction

The xenoestrogen bisphenol A (BPA), a food contaminant with endocrine disrupting activity, is widely used to manufacture consumer products including food and beverage containers, cigarette filters, thermal (carbonless) receipts, and paper currency [1]. It has been reported that over 2.7 million metric tons of BPA are produced annually and therefore BPA becomes the highest volume chemicals produced worldwide [2]. BPA can leach from the polymers into food and water under normal conditions; furthermore, exposure to elevated temperatures (boiling, heating) greatly increases its rate of migration [3]. Due to an increase in products based on epoxy resins and polycarbonate plastics, exposure of human beings to BPA via food consumption and environmental intakes has increased in recent years [4]. Numerous studies have suggested that human exposure to BPA is widespread, which is evidenced by its presence in urine, blood, fetal tissues, and amniotic fluid [2,5]. For example, BPA has been detected in 100% of urine samples from Chinese children [6] and in 96% of urine samples from American [7]. Studies suggested that BPA is significantly correlated with health outcomes, such as diabetes, cardiovascular disease, increased inflammation, and cancer [8].

Lung cancer is one of the leading causes of cancer deaths worldwide [9], killing an estimation of 1.4 million people annually. Furthermore, it is also one of the most difficult cancers to treat because there are very limited treatment options for advanced lung cancer. Non-small cell lung cancer (NSCLC) constitutes approximately 80–85% of all lung cancer cases [10]. Compelling
evidence from clinical studies indicates that more than 70% of NSCLC patients show metastases to the regional lymph nodes or to distant sites at the initial presentation [9]. Several identified several factors associated with risk of lung cancer besides the smoking, such as air inhalation [11]. Previous studies have indicated that permeation and absorption of BPA through inhalation is an important human exposure pathway [12]. Therefore, the roles of BPA on the tumorigenesis and development of NSCLC cancer should be investigated.

More recently, several epidemiologic and clinical studies have provided evidence of a role for estrogens in the genesis and progression of NSCLC [13]. Estrogen can promote the lung cancer growth and metastasis both in vitro and in vivo [14,15]. Further, new emerging estrogenic receptors, such as G-protein-coupled estrogen receptor (GPER) and estrogen-related receptor (ERR) have been detected in various NSCLC cell lines [16,17]. Therefore it is reasonable to assume that BPA can influence the progression of NSCLC due to all these estrogen receptors can mediate estrogenic signaling of BPA.

Therefore, the present study was designed to investigate effects of BPA on migration and invasion of NSCLC cancer. Further, the downstream signal pathways of BPA induced migration of NSCLC cancer cell lines were further investigated. To our knowledge, this is the first study to illustrate effects of BPA on NSCLC metastasis.

2. Materials and methods

2.1. Chemicals and reagents

IC 182,780 (ICI, ER antagonist) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PD 98059 (PD, MAPK/ERK kinase agonist) and AG 1478 (AG, EGFR antagonist) were purchased from Selleck Chemicals (Houston, TX, USA). G15 (GPR30 antagonist) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BPA was dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution, and stored at −20 °C. Primary antibodies against matrix metalloproteinase-2 (MMP-2), MMP-9, p-ERK1/2, ERK1/2, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All compounds were solubilized in DMSO with the concentrations of DMSO in the assay were less than 0.5% (v/v). Medium containing 0.5% DMSO was used as the control.

2.2. Cell line and culture

Human cell line A549 was obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 with 10% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin at 37 °C in a humidified 5% CO2 atmosphere. Both the plastic items used for the experiments and the water used to prepare the reagents, which were pretreated by enhanced sonoochemical degradation to reduce any potential background BPA [18,19]. Twenty-four hours before the experiments, the medium was removed and replaced with DMEM without phenol red supplemented with 5% dextran-coated, charcoal-treated FBS (5% DC-FBS) to exclude estrogenic effects caused by the medium. Then, cells were plated in the same medium and allowed to attach overnight.

2.3. Cell viability assay

Cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) assay. Briefly, cells (1 × 104) were seeded into each well of a 96-well plate. After 24 h, the cells were treated with BPA at concentrations from 10−8 M to 10 μM for 24 h. After the treatment, medium containing BPA was removed, cells were washed twice with phosphate-buffered saline (PBS), and 100 μL of 0.25 mg/mL MTT in medium culture was added to each well. The plate was incubated for 4 h at 37 °C. Then, culture medium was removed, and DMSO (100 μL) was added into each well to dissolve the dark blue crystal. The absorbance was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA, USA). Cell viability was expressed as a percentage of the absorbance value of control cultures. The experiments were repeated six times.

2.4. Wound healing assay

Confluent monolayers of A549 cells were scratched with three separate wounds by the use of a sterile 200 μL pipette tip. Subsequently, the culture was washed twice with serum-free medium and replaced with fresh medium. The migration ability of the cells was assessed by measuring the width of the monolayer wound at the indicated times after scraping. Pictures were taken just above and just below each line to ensure that the line just appears in each picture. The wound border was measured in nine random fields.

2.5. In vitro migration/invasion assay

The polycarbonate filters (8 μm pore size, Corning) pre-coated with Matrigel Matrix (BD Biosciences) were used for invasion assay, and uncoated filters were used for migration assay. Cells (1 × 104) in 300 μL medium (containing 0.1% FBS) treated with or without BPA were seeded in the upper chamber. Then, 600 μL medium with 10% FBS was added to the lower chamber and served as a chemotactic agent. After 48 h incubation for migration, the cells migrated and adhered onto the lower chamber were fixed in 4% paraformaldehyde for 20 min, stained with hematoxylin and counted under upright microscope (5 fields per chamber). To quantify invasion, cells in the upper chamber were fixed in 4% paraformaldehyde for 20 min. Then, the Matrigel was mechanically removed from the filter with a cotton swab. Cells adhering to the under-side of the filter were stained with hematoxylin and counted under upright microscope (5 fields per chamber). Each migration and invasion assay was repeated in three independent experiments.

2.6. Western blot analysis

Cells were treated with or without BPA for the indicated times before lysed in cell lysis buffer, and then lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer. Aliquots of protein were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated with the primary antibody at 1:1000 dilution overnight at 4 °C and then incubated with a horseradish peroxidase-conjugated secondary antibody at 1:1000 dilution for 2 h at room temperature, and detected with the Western Lightning Chemiluminescent detection reagent (Perkin-Elmer Life Sciences, Wellesley, MA).

2.7. Quantitative real-time PCR

A549 cells (1 × 104) were plated on 6-well plates. After treated with BPA for 24 h, cells were washed twice with ice-cold PBS. Total mRNA was extracted with TRIZOL reagent. First strand of cDNA was generated from 2 μg total RNA using oligo-dT primer and
Superscript II Reverse Transcriptase (GIBCO BRL, Grand Island, NY, USA). Quantitative real-time PCR was run on an iCycler (Bio-rad, Hercules, USA) using validated primers and SYBR Premix Ex Taq II (Takara, Japan) for detection. The cycle number when the fluorescence first reached a preset threshold (Ct) was used to quantify the initial concentration of individual templates for expression of mRNA of genes of interest. Primer pairs were as follows: MMP-2 forward5’-ACA ATG AGG TGA AGA AGA AA A TGG A-3’ and reverse 5’-AGG TAA TAG GCA CCC TTG AAG AAG TA-3’; MMP-9 forward5’-CAC TGT CCA CCC CTC AGA GC-3’ and reverse 5’-GCC ACT TGT CGG CCG TAG G-3’; GAPDH, forward 5’-GCA CGG TCA AGG CTG AGA AC-3’ and reverse 5’-TGG TGA AGA GCC CAG TGG A-3’. Transcripts of the housekeeping gene GAPDH in the same incubations were used for internal normalization.

2.8. Statistical analysis

All values were reported as mean ± SD of three independent experiments unless otherwise specified. Data were analyzed by two-tailed unpaired Student’s t-test between two groups and by one-way ANOVA followed by Bonferroni test for multiple comparison involved. The statistical analyses were performed using SPSS 13.0 for Windows. A P-value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of BPA on cell viability of A549 cells

To evaluate the effects of BPA on the cell viability of NSCLC cancer, A549 cells were treated with various concentrations of BPA ranging from 10^-10 to 10^-4 M for 24, 48 and 72 h. As shown in Fig. 1A, 10^-10 to 10^-5 M BPA had limited effects on the proliferation of A549 cells. But 10^-4 M BPA can inhibit the cell proliferation via a time-dependent manner. Interestingly, we found that BPA treatment significantly altered the morphology of A549 cells. As shown in Fig. 1B, A549 cells treated with BPA, particularly for 10^-5 M BPA, for 72 h resulted in several morphological changes, with an accompanying loss of an adherent phenotype, decreased cell-to-cell contacts and the induction of a fibroblast-like state.

3.2. Effects of BPA on cell motility of A549 cells

The morphological changes suggested that BPA may modulate the motility of A549 cells, then it was further investigated by use of wound healing and transwell migration/invasion assays. We found that treatment with 10^-5 M BPA for either 48 or 72 h significantly increased wound closure as compared to the control group (Fig. 2A). Meanwhile, the transwell migration and invasion assays showed that 10^-3 M BPA significantly promoted the migration and invasion of A549 cells in vitro, as evidenced by a significant increase in the number of cells that migrated (Fig. 2B) and invaded through the polycarbonate filter (P < 0.01). Collectively, our data revealed that BPA could significantly promote the migration and invasion of lung cancer cells.

3.3. BPA upregulated the expression of MMPs

The matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that play an important role in the migration and invasion of various types of cancer cell [20]. We then detected the mRNA and protein expression of MMP-2 and MMP-9 in A549 cells treated with BPA for the indicated times. The results revealed that BPA can upregulate both the mRNA (Fig. 3A) and protein (Fig. 3B) levels of MMP-2 and MMP-9 via a concentration-dependent manner. Further, treatment of A549 cells with 10^-3 M BPA increased the expression of both MMP-2 and MMP-9 via a time-dependent manner.

3.4. GPER mediated the BPA induced upregulation of MMPs

Previous studies indicated that both classic estrogen receptor α/β (ERα/β) and GPER can mediate the estrogenic effects of BPA and other environmental estrogens [21]. Therefore, the roles of GPER and ERα/β in BPA induced MMPs were investigated. It was found that both ICI 182,780 (the specific inhibitor of ERα/β) and G15 (the specific inhibitor of GPER) had no obvious effects on the expression of MMP-2 and MMP-9. However, G15, but not ICI 182,780, significantly abolished the 10^-5 M BPA induced upregulation of MMP-9 and MMP-2 (Fig. 4). It suggested that GPER mediates the BPA induced upregulation of MMPs in lung cancer cells.

3.5. BPA induced rapid phosphorylation of ERK1/2 via GPER/EGFR

Recent studies indicated that BPA could modulate the activities of ERK1/2 via GPER/EGFR signals [22,23]. Therefore, the effects of BPA on the phosphorylation of ERK1/2 were measured by western blot analysis. As shown in Fig. 5A, BPA caused a rapid (10 min) phosphorylation (activation) of ERK1/2 in A549 cells (Fig. 5A). Maximum phosphorylation of ERK1/2 was reached at 10–15 min. Further, both G15 (the specific inhibitor of GPER) and AG 1478 (the specific inhibitor of EGFR) significantly abolished the BPA induced activation of ERK1/2 in A549 cells (Fig. 5B). Our data revealed that BPA could phosphorylate the ERK1/2 via GPER/EGFR signals in lung cancer cells.

3.6. GPER/EGFR/ERK1/2 mediated BPA induced upregulation of MMPs and cell migration

It was suggested that the ERK activation is necessary for MMP-9 expression in cancer cells [24,25]. Therefore, the roles of GPER
mediated activation of ERK1/2 in BPA induced upregulation of MMPs and cell migration were investigated. We found that when EGFR antagonist AG 1478 or the mitogen-activated protein kinase MEK antagonist PD 98059 or the GPR30-specific antagonist G15 has no effects on the migration of A549 cells treated with antagonist alone. However, all inhibitors successfully abolished stimulatory effects of $10^{-5}$ M BPA on the in vitro migration of A549 cells (Fig. 6A). Furthermore, up regulation of MMP-2 and MMP-9 caused by exposure to BPA was abolished in the presence of G15, PD 98059, or AG 1478 (Fig. 6B), suggesting that GPER/EGFR/ERK1/2 signaling is required for the upregulation of MMPs and consequently for BPA induced A549 cell migration.

Fig. 2. Effects of BPA on cell motility of A549 cells. A. Confluent monolayers of A549 cells were scraped by a pipette tip to generate wounds and then were cultured in the presence or absence of $10^{-5}$ M BPA. Representative images of wounds at 0, 24, 48, and 72 h were observed. A549 cells were allowed to migrate transwell chambers or spread through the matrix gel and into the under-side of the filter for 24, 48, and 72 h in the presence or absence of $10^{-5}$ M BPA. Then, the migrated or invaded cells were fixed, stained, and photographed. The number of migrated (B) or invaded (C) cells were compared with the control. Data are presented as means ± SD of three independent experiments. *P < 0.01 compared with control.

Fig. 3. Effects of BPA on mRNA and protein expressions of MMP-2 and MMP-9. A. A549 cells were treated with various concentrations of BPA ($10^{-6}$ to $10^{-9}$ M) for 24 h, and then, the mRNA levels of MMP-2 and MMP-9 were measured by qRT-PCR. B. A549 cells were treated with various concentrations of BPA ($10^{-6}$ to $10^{-9}$ M) for 48 h, and then, the protein levels of MMP-2 and MMP-9 were measured by western blot analysis. C. A549 cells were treated with $10^{-5}$ M BPA for 0 to 72 h, and then the protein levels of MMP-2 and MMP-9 were measured by western blot analysis.
4. Discussion

More and more studies indicated that BPA can modulate the tumorigenesis and development of cancer cells, particularly for estrogenic related cancers [22,26]. Estrogen receptors can be detected in lung cancer cells and promote its development [27], and there is no study about the effects of BPA on lung cancer cells. The present study revealed that BPA could trigger the in vitro migration and invasion of lung cancer A549 cells. This effect was mediated by new emerging estrogenic receptor GPER while not the classic ERα/β. The downstream signals GPER/EGFR/ERK1/2 was responsible for the BPA-induced upregulation of MMPs and then in vitro migration of lung cancer A549 cells. To our knowledge, this is the first study illustrating that BPA can promote the development of lung cancer by triggering its metastasis.

There is limited study about the relationship between BPA and cancer. Few epidemiological studies have linked BPA to different types of cancer. For example, BPA is considered as a potential risk factor to breast cancer [28]. Low BPA doses can accelerate mammary tumorigenesis and metastasis in MMTV-erbB2 mice [29]. In the present study, we used phenol red–free growth medium and DCC-FBS to eliminate the effects of estrogen and found that BPA less than 10^{-4} M has limited effects on the cell proliferation, while even 10^{-5} M BPA can significantly upregulate the mRNA and protein levels of MMPs. Further, treatment with 10^{-5} M BPA for 72 h can induce cell migration 1.5-fold and cell invasion 1.4-fold greater than the control group. Our results were consistent with a recent study, which revealed a stimulatory effect of BPA on ovarian cancer cell migration [22]. It was also supported by the results that exogenous and endogenous estrogen plays a positive role in development of lung cancer, especially adenocarcinoma [30]. Therefore, more attention should be paid to the inhalation intakes of BPA and air pollution due to the direct interaction between BPA and lung cells.

The stimulatory effects of BPA on cell migration were accompanied by upregulation of the migration-related factors MMP-2 and MMP-9. It was in line with the recent study that BPA induced migration of OVCAR-3 ovarian cancer cell was accompanied by upregulation of the migration-related factors MMP-2, MMP-9 and N-cadherin [22]. Another study also demonstrated that BPA stimulates the secretion of MMP-9 in human granulosa-lutein cells [31]. Considering that MMPs can trigger the migration and invasion of various types of cancer cell [20], furthermore, the upregulation of MMP-2 and MMP-9 are associated with poor prognosis of lung cancer [32], our data revealed that BPA can stimulate the lung cancer cell migration by influencing the expression levels of MMP-2 and MMP-9.

The estrogenic signals are classically mediated by ERα/β that act as transcription factors to modulate activities of target genes by interacting with several DNA response elements. Recent study revealed that GPER potentially mediate rapid E2-dependent proliferation in cancer cells via directly or indirectly modulating cellular responses, such as calcium mobilization, kinase activation, and nitric oxide production with a slower time course [33–35]. In the present study, GPER, while not ERα/β, is found to be mediated the BPA induced migration, since antagonist of GPER (G15) was...
able to block the effects of BPA on up regulation of MMPs. These results are similar to recent study that GPER mediates the BPA induced rapid activation in breast cancer cells [36] and testicular seminoma cells [37]. The results of the present study revealed that BPA-stimulated lung cancer cell migration was through GPER while not ERα/β.

Previous studies reported that ERK can be rapidly activated by GPER and then mediate the non-genomic estrogen responses induced by BPA [36,38,39]. There were accumulating evidences suggested that MAPK/ERK plays a central role in the regulation of cancer cell migration [40,41]. In the present study, BPA can stimulate rapid (10 min) phosphorylation of ERK1/2 in A549 cells, which is consistent with the results of previous studies performed in cancer cells [42,43]. The activation of ERK1/2 by BPA was induced via GPER/EGFR signals, since both G15 and AG1478 effectively abolished this effect. Previous reports also showed similar findings in endometrial cancer, ovarian cancer, thyroid cancer, and breast cancer-associated fibroblasts ([34,44]; [45]), which might suggest that the activation of estrogen/GPER/ERK signaling might be a common mechanism in estrogen-related tumors [46].

Further, our study revealed that GPER/EGFR/ERK1/2 mediated the BPA induced cell migration. That estrogen/GPER/ERK signaling had a potential role in the cell invasion, which was observed in breast cancer cells, such as MDA-MB-468 and MDA-MB–436 cells [46]. Our data also were consistent with previous study of the roles of ERK in migration stimulate by E2 in endometrial [47] and other cancer [48] cells. Moreover, inhibition of GPER/EGFR/ERK1/2 signals attenuated the stimulatory effects of BPA on the expression levels of MMP-2 and MMP-9. This was also confirmed by previous studies that MAPK/ERK is necessary for MMP-9 expression in cancer cells [24,25].

In conclusion, the current study characterized that BPA has limited effects on the cell viability but can significantly promote the in vitro migration and invasion of human lung cancer A549 cells via upregulation of MMPs by dose- and time-dependent manners. Further, GPER/EGFR/ERK1/2 signals mediated the BPA induced cell migration and upregulation of MMPs. This study provided new sight for the first time to tumorigenesis and metastasis for lung cancer development stimulated by BPA and possibly other environmental estrogens.

References


